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Biosynthetic Mechanisms

Production of the Antifungal Isochromanone Ajudazols A and B in *Chondromyces crocatus* Cm c5: Biosynthetic Machinery and Cytochrome P450 Modifications**

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Over the last 25 years, myxobacteria have emerged as a rich source of natural products with potent biological activities.[1] Of particular note is the strain *Chondromyces crocatus* Cm c5, responsible for biosynthesis of at least six distinct classes of secondary metabolites, many of which include unique structural elements.[2–5] Among these metabolites are the antifungal ajudazols A (**1**) and B (**2**) (Figure 1), potent inhibitors of mitochondrial electron transport.[6] The ajudazols are novel isochromanone derivatives that incorporate an extended side chain containing an oxazole ring, a *Z*,*Z* diene, and a 3-methoxybutenoic acid amide.[2] Whereas ajudazol A (**1**), the major metabolite, contains an *exo*-methylene functionality at C15, ajudazol B (**2**) has a methyl group at this position. Although the shared backbone of the ajudazols could be predicted straightforwardly to arise from a mixed system consisting of type I polyketide synthase (PKS) and non-ribosomal polypeptide synthetase (NRPS) multienzymes,[7] the origin of several functional groups, including the isochromanone and the *exo*-methylene group of ajudazol A, was not obvious from considerations of classical assembly-line biosynthesis. We aimed, therefore, to identify the ajudazol gene cluster in *C. crocatus* Cm c5 in order to study the underlying biosynthetic processes and to enable the directed generation of novel ajudazol analogues.

As the amino acids glycine and serine are apparently incorporated into the ajudazols, we anticipated that the biosynthetic machinery would include two NRPS modules. We therefore attempted to identify the gene cluster in *C. crocatus* Cm c5 by inactivating NRPS adenylation (A) domains and then screening for the loss of ajudazol production. Internal A-domain sequences (motifs A3–A10)[8] were amplified from the genomic DNA of Cm c5 using degenerate primers. Sequencing revealed 11 unique A-domain fragments, all of which were used for insertional mutagenesis in Cm c5 (see the Supporting Information). Insertion of one such fragment abolished production of both ajudazols A and B. A probe based on this fragment was then used to screen a 2304 clone chromosomal library of *C. crocatus* Cm c5.[9] End-sequencing of a positive cosmid D:D11 revealed sequences with homology to PKS genes. We identified the remainder of the cluster on overlapping cosmids C:B8 and B:A15 by designing probes against both ends of D:D11, and rescreening the chromosomal library. Sequencing of the three cosmids (104292 bp in total) revealed the ajudazol biosynthetic gene cluster, which spans a contiguous stretch of 70839 bp on the *C. crocatus* genome (the gene cluster has been deposited in the EMBL database under accession number AN946600).

The average (guanine + cytosine) content of the gene cluster is 70.3%, which is typical for myxobacteria.[10] The cluster comprises 12 genes, including eight type I PKS (*ajuA*–*ajuC*, *ajuE*–*ajuH* and *ajuK*), one NRPS (*ajuD*), and one hybrid NRPS–PKS (*ajuL*) (Table 1). As assigning gene boundaries based on ribosome binding sites is often difficult in myxobacteria, the start codons for many genes were identified by sequence alignment of the translated N-terminal docking domain regions.[11] Sequence analysis of the NRPS modules revealed that the insertional mutagenesis had occurred within the A-domain sequence of *ajuD*.

The mixed PKS–NRPS pathway to the ajudazols reveals a high level of colinearity between the gene complement and the required series of biochemical transformations, which is atypical for myxobacterial systems.[12,13] The only departure from this colinear relationship is the location at the end of the cluster of genes *ajuK* and *ajuL*, which encode the first two biosynthetic proteins (Figure 1). As in other myxobacterial systems, AjuK contains an unusual first module incorporating domains involved in both chain initiation and extension.[7,14] The module also contains a SAM-dependent *O*-methyltransferase which is predicted to methylate the enol form of the diketide intermediate; this mechanism was also postulated for generation of the β-methoxyacrylate functionalities of myxothiazol and melithiazol.[15,16] The remaining steps in the assembly of the ajudazol backbones can be assigned readily to the constituent modules of subunits AjuA–AjuH, which incorporate AT or A domains of appropriate specificity, and the correct complement of modifying activities. The only exceptions are required DH domains, as these activities are absent from modules 3 and 5; the DH present in module 12

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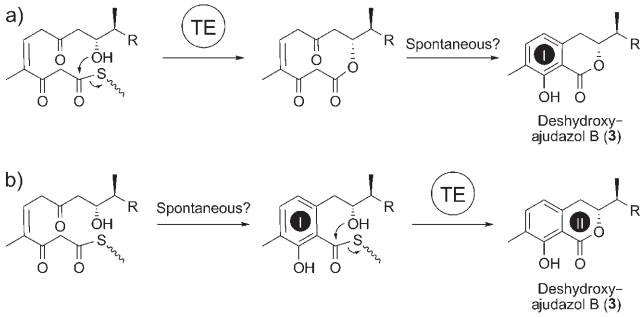
Figure 1. Biosynthesis of the ajudazols in *C. crocatus* Cm c5. a) Organization of the biosynthetic gene cluster. b) Model for biosynthesis on the ajudazol mixed PKS–NRPS synthetase. The structures of ajudazols A (**1**) and B (**2**) are shown. The DH domains present in modules 4, 6, and 13 are presumed to act iteratively (see text), while that indicated with an asterisk is assumed to be inactive.

Table 1: Identity and proposed function of proteins encoded within the ajudazol biosynthetic gene cluster.

PKS/NRPS portion		
Protein (gene)	Size (in Da, bp)	Protein domains (position in the sequence)
AjuA (*ajuA*)	230480/6519	KS (87–1367), AT (1695–2586), DH (2763–3279), ER (4359–5280), KR (5307–5844), ACP (6141–6339)
AjuB (*ajuB*)	361780/10212	KS (123–1401), AT (1722–2604), KR (3471–4008), ACP (4326–4536), KS (4596–5883), AT (6204–7086), DH (7287–7818), KR (8970–9507), ACP (9819–10023)
AjuC (*ajuC*)	236540/6669	KS (108–1401), AT (1710–2607), DH (2811–3324), ER (4485–5425), KR (5481–6018), ACP (6324–6525)
AjuD (*ajuD*)	155250/4227	HC (201–1503), A (1539–3869), Ox (3213–3724), PCP (3939–4134)
AjuE (*ajuE*)	232410/6537	KS (27–1290), AT (1605–2502), DH (2694–3204), ER (4356–5289), KR (5352–5889), ACP (6180–6381)
AjuF (*ajuF*)	167740/4743	KS (105–1389), AT (1701–2592), KR (3537–4074), ACP (4380–4584)
AjuG (*ajuG*)	312570/8841	KS (111–1392), AT (1695–2592), ACP (2928–3132), KS (3198–4491), AT (4806–5697), DH (5877–6426), KR (7539–8097), ACP (8400–8607)
AjuH (*ajuH*)	172740/4743	KS (108–1392), AT (1704–2598), DH (2817–3345), ACP (3828–4029), TE (4167–4842)
AjuK (*ajuK*)	312570/8841	ACP (75–276), KS (348–1605), AT (1848–2736), AT (3171–4065), *O*-MT (4332–5378), ACP (5382–5529)
AjuL (*ajuL*)	337710/9384	C (174–1497), A (1521–4278), *N*-MT (2933–4149), PCP (4344–4539), KS (4623–5895), AT (6207–7125), KR (8121–8658), ACP (8955–9159)
Post-assembly line enzymes		
Protein (gene)	Size (in Da, bp)	Homologue, origin (identity [%], similarity [%]; accession no.)
AjuI (*ajuI*)	51700/1377	cytochrome P450 TaH, *Myxococcus xanthus* (37, 58; CAB40542)
AjuJ (*ajuJ*)	54910/1470	cytochrome P450 TaH, *Myxococcus xanthus* (36, 56; CAB4054)

lacks several conserved active-site residues and so is presumed to be inoperative. As postulated for other myxobacterial PKS, the functions of the missing DHs may be complemented by the iterative action of DH domains in the downstream modules 4, 6, and 13.[14,17,18] The ajudazols contain two *Z* double bonds and one *E*; as for other myxobacterial systems,[17] the predicted stereochemistry of the precursor hydroxyl group (see the Supporting Information)[19] does not correlate in each case with the observed double-bond geometry, as the B-type hydroxy functions expected in modules 5 and 6 both give rise to Z (*cis*) double bonds. Therefore, the mechanism for controlling the double-bond stereochemistry in these cases remains unclear.

In general, the biosynthesis of aromatic structures by bacteria is accomplished by type II or type III PKS systems, and not by modular type I PKS. Among the aromatic moieties of complex polyketides, only the chromone of stigmatellin is thought to arise from catalysis by a domain integral to a modular PKS, a dedicated C-terminal cyclase.[20] An analogous cyclase to form the ajudazol isochromanone is not present at the end of PKS subunit AjuH, which terminates instead in a thioesterase (TE) domain. Such domains are typical of both PKS and NRPS systems, and catalyze chain release by macrolactonization or hydrolysis.[21] This observation suggests two alternative mechanisms for chain release coupled to isochromanone formation, to yield the putative intermediate deshydroxyajudazol B (**3**) (Scheme 1): a) TE-catalyzed attack of the C9 hydroxy group onto the ACP-bound thioester to give the free ten-membered lactone, followed by C2–C7 aldol addition and ring I aromatization; or b) aldol addition/aromatization to form ring I while the intermediate remains tethered to the ACP, followed by TE-catalyzed lactonization and chain release to yield ring II.



Scheme 1. Proposed mechanisms for formation of the isochromanone ring system and chain release. a) The TE catalyzes lactone ring formation, which is followed by aldol addition and aromatization of ring I. b) Aldol addition and aromatization occur to generate ring I, followed by TE-catalyzed lactonization and chain release to afford ring II.

Surprisingly, sequence analysis shows that the ajudazol TE exhibits highest homology to discrete (type II) TE domains present in NRPS and PKS systems, rather than to typical type I TEs (see the Supporting Information). Type II TEs are postulated to regenerate stalled assembly lines through hydrolytic release of misacylated,[22,23] and in the case of NRPS, misaminoacylated[24] substrates from the integral carrier proteins. As neither the expected specificity of the *aju*TE (for simple fatty acyl chains or amino acids) nor the chemistry of chain release correlates with the proposed mechanisms for isochromanone formation, off-loading of the chain may be a spontaneous process. Indeed, the full functionality required to generate the isochromanone is only installed following the last condensation reaction (Figure 1). In this model, the TE would serve to "chaperone"

the folding process, analogous to the function of cyclase domains in the biosynthesis of aromatic polyketides.[25] In either case, a compound with the predicted mass of deshydroxyajudazol B (**3**) (Schemes 1 and 2) is present in extracts of wild-type *C. crocatus* (see the Supporting Information), supporting its intermediacy in the biosynthesis.

The biosynthesis of ajudazols A and B also requires several post-PKS reactions in order to generate the hydroxy function at C8 and the C15 *exo*-methylene of ajudazol A (**1**). The only candidate genes in the cluster to encode these enzymes were *ajuI* and *ajuJ*, embedded between PKS genes *ajuH* and *ajuK*. AjuI shows highest homology to P450 enzymes from the myxobacteria *Myxococcus xanthus*[26,27] and *Sorangium cellulosum*,[14,28] while the closest homologues to AjuJ are uncharacterized P450s from the cyanobacteria *Nostoc punctiforme* PCC 73102 and *Nodularia spumigena* CCY 9414; the proteins exhibit 38% mutual sequence homology. To attempt to assign roles to AjuI and AjuJ, the respective genes were inactivated by insertional mutagenesis in the Cm C5 chromosome (see the Supporting Information).

Analysis of extracts of the *ajuI* mutant by HPLC-MS revealed the complete absence of ajudazol A (**1**), and a corresponding significant increase in production of ajudazol B (**2**) (Figure 2). The *ajuJ* mutant did not produce either ajudazols A or B. However, inspection of the HPLC-MS data from the mutant revealed the previously uncharacterized compound **4** (m/z $[M+H]^+ = 575.3$) produced at a yield of $0.33\ mg\,L^{-1}$; reanalysis of data from the wild-type strain showed that **4** was also present, although at much lower levels (Figure 2). The compound was substantially purified, and its structure was analyzed by NMR spectroscopy and high-resolution mass spectrometry (see the Supporting Information). This analysis identified the metabolite as a deshydroxy derivative of ajudazol A, lacking the OH functionality at C8 (Table 2).

Taken together, these results allow us to propose a mechanism for the post-PKS tailoring of the ajudazol structures (Scheme 2), although we cannot formally exclude that oxidative modification takes place on an ACP-bound intermediate. The product released from the multienzyme AjuH is likely to be deshydroxyajudazol B (**3**), which becomes a substrate for both P450 enzymes AjuI and AjuJ. If AjuJ acts first, its product ajudazol B (**2**) is no longer a substrate for AjuI. However, if AjuI operates first, the resulting deshydroxyajudazol A (**4**) is accepted by AjuJ, generating ajudazol A (**1**). P450-mediated desaturation, as proposed here for AjuI, is not uncommon in eukaryotic primary metabolism and detoxification pathways,[29] and has also been shown to occur in the biosynthesis of the fungal metabolites aflatoxin and sterigmatocystin,[30] as well as in flavone biosynthesis in plants.[31] However, this is, to our knowledge, the first example from bacterial metabolism. In the absence of AjuJ, as in the *ajuJ* mutant, the strain accumulates deshydroxyajudazol A (**4**), whereas if AjuI is absent, the deshydroxyajudazol B (**3**) is converted to ajudazol B (**2**) by AjuJ. Presumably, AjuI is a more efficient catalyst than AjuJ, accounting for the significantly higher proportion of ajudazol A (**1**) in extracts of native *C. crocatus*.

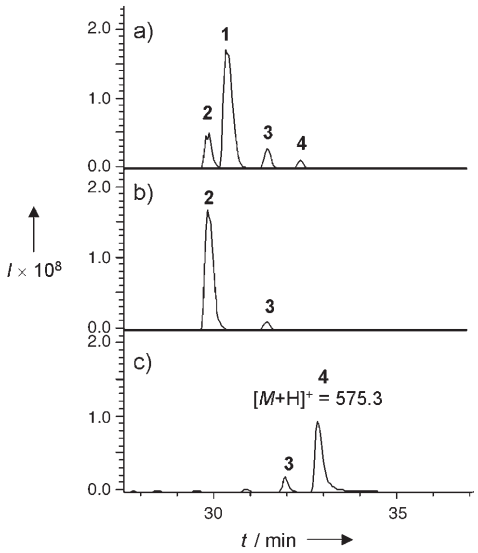


Figure 2. HPLC-MS chromatogram of culture extracts of a) *C. crocatus* Cm c5 wild type and the mutants in b) *ajuI* and c) *ajuJ*. Shown is the base peak chromatogram (BPC) in the mass range 575.0–594.0. Mutant *ajuJ* produces increased amounts of deshydroxyajudazol A (**4**) (m/z $[M+H]^+ = 575.3$), relative to the wild-type strain (ajudazol A (**1**): $[M+H]^+ = 591.3$; ajudazol B (**2**): $[M+H]^+ = 593.3$; deshydroxyajudazol B (**3**): $[M+H]^+ = 577.3$).

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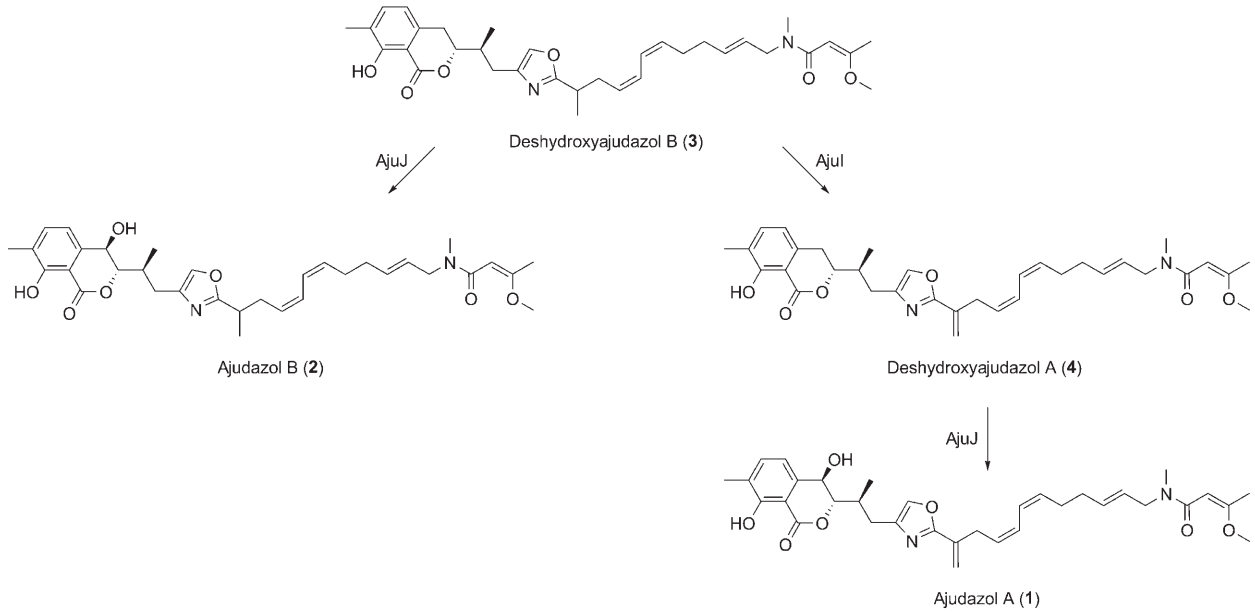
Table 2: Subset of the NMR spectroscopic data for ajudazol A (**1**) and deshydroxyajudazol A (**4**).

	Ajudazol A (**1**)[a]				Deshydroxyajudazol A (**4**)[b]			
Atom	δ_H	M	J	δ_C	δ_H	M	J	δ_C
6	6.95	d	7.6	116.63	6.72	d	7.3	118.63
7	–	–	–	140.21	–	–	–	108.59
8	4.79	d(d)	6.7	63.87			–	30.4
8-OH	6.01	d	6.5	–				
9	4.39	dd	6.7, 5.6	87.26	4.5	ddd	9.1, 6.4, 6.4	84.69
10			–	33.29			–	34.75
15	–	–	–	134.36	–	–	–	136.12
15-CH_a			br	117.42			br	118.55
15-CH_b			br				br	118.55
16_a, 16_b	3.27	d	7.8 br	30.03	3.35	d	7.31	31.21

[a] NMR data reproduced from Jansen et al.[2] [b] In $[D_6]$MeOH at 500 MHz.



Scheme 2. Proposed mechanism for the post-PKS modifications catalyzed by the cytochrome P450 enzymes AjuI and AjuJ.

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